



Adipose tissue–derived mesenchymal stem cells promote the vascularization of pancreatic islets transplanted into decellularized pancreatic skeletons

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ABSTRACT

We have recently developed a model of pancreatic islet transplantation into a decellularized pancreatic tail in rats. As the pancreatic skeletons completely lack endothelial cells, we investigated the effect of co-transplantation of mesenchymal stem cells and endothelial cells to promote revascularization.

Decellularized matrix of the pancreatic tail was prepared by perfusion with Triton X-100, sodium dodecyl sulfate and DNase solution. Isolated pancreatic islets were infused into the skeletons via the splenic vein either alone, together with adipose tissue–derived mesenchymal stem cells (adMSCs), or with a combination of adMSCs and rat endothelial cells (rat ECs). Repopulated skeletons were transplanted into the subcutaneous tissue and explanted 9 days later for histological examination. Possible immunomodulatory effects of rat adMSCs on the survival of highly immunogenic green protein–expressing human ECs were also tested after their transplantation beneath the renal capsule. The immunomodulatory effects of adMSCs were also tested in vitro using the Invitrogen Click-iT EdU system.

In the presence of adMSCs, the proliferation of splenocytes as a response to phytohaemagglutinin A was reduced by 47% (the stimulation index decreased from 1.7 to 0.9, $P = 0.008$) and the reaction to human ECs was reduced by 58% (the stimulation index decreased from 1.6 to 0.7, $P = 0.03$). Histological examination of the explanted skeletons seeded only with the islets showed their partial disintegration and only a rare presence of CD31-positive cells. However, skeletons seeded with a combination of islets and adMSCs showed preserved islet morphology and rich vascularity. In contrast, the addition of syngeneic rat ECs resulted in islet-cell necrosis with only few endothelial cells present. Live green fluorescence–positive endothelial cells transplanted either alone or with adMSCs were not detected beneath the renal capsule.

Though the adMSCs significantly reduced in vitro proliferation stimulated by either phytohaemagglutinin A or by xenogeneic human ECs, in vivo co-transplanted adMSCs did not suppress the post-transplant immune response to xenogeneic ECs. Even in the syngeneic model, ECs co-transplantation did not lead to sufficient vascularization in the transplant area. In contrast, islet co-transplantation together with adMSCs successfully promoted the revascularization of extracellular matrix in the subcutaneous tissue.

1. Introduction

The isolation of pancreatic islets dispersed within the pancreatic exocrine tissue completely disrupts islet microvasculature [1,2] and the natural extracellular matrix that is important for islet interaction within

the islet transplant site [3,4]. When islets are administered intravascularly (such as into the liver portal system), they are exposed to direct contact with blood components, with a subsequent instant blood-mediated inflammatory reaction that initiates coagulation, leads to hypoxic stress and local inflammation, and activates immune-mediated

Abbreviations: adMSCs, Adipose tissue-derived mesenchymal stem cells; ECs, Endothelial cells; GFP, Green fluorescence protein; VEGF, Vascular endothelial growth factor; EdU, Nucleoside analog 5-ethynyl-2'-deoxyuridine; PHA, Phytohemagglutinin A; PBS, Phosphate buffered saline..

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reactions [5,6].

The instant blood-mediated inflammatory reaction may be partially obviated by systemic or local administration of anticoagulation or anti-inflammatory drugs [7,8] or completely prevented by placing the islet graft in an extravascular space [9–11], encapsulating islets with extracellular proteins [12], or using decellularized organs as scaffolds for cell therapies [13,14]. Decellularized pancreas could be a suitable scaffold as it contains native extracellular proteins. However, it is limited by its low immediate oxygen supply and a rather slow revascularization rate due to the absence of endothelial cell progenitors [15].

2. Objective

We have recently developed a rat model of pancreatic islet transplantation into a decellularized pancreatic tail with preserved molecular matrix structures that are important for islet attachment and engraftment within the host tissue [16]. As the skeletons are completely rid of endothelial cells (ECs), in our previous study we placed them in a highly vascularized omental flap. In the current study we investigated the effect of adipose tissue-derived mesenchymal stem cells (adMSCs) [17–19] co-transplanted in order to promote revascularization in the subcutaneous space. As the adMSCs show multiple supportive and immunomodulatory effects [20,21], we also tested their ability to modify in vitro and in vivo the immune response to highly immunogenic ECs.

3. Materials and methods

3.1. Experimental animals

Adult male Lewis rats (Charles River, Germany) were used as donors of intraabdominal fat, spleens, and pancreases for decellularization and pancreatic islet isolations. Lewis rats also served as recipients in transplantation experiments. All experimental protocols were approved by the Experimental Animals Welfare Committee of the Institute for Clinical and Experimental Medicine and the Ministry of Health of the Czech Republic (approval no. 36/2018) in accordance with the European Communities Council Directive 86/609/EEC.

3.2. Isolation and cultivation of rat adMSCs

AdMSCs were isolated as described previously [22]. Briefly, samples of retroperitoneal and epididymal fat from Lewis rats were digested with collagenase (1 mg/ml in Hanks balanced salt solution, 30 min, 37 °C; cat. no. C9263, Merck, Darmstadt, Germany). Digestion was stopped with 4% fetal bovine serum (FBS, Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS). Tissue was disintegrated by passage through a 230-µm strainer and washed in PBS. AdMSCs were separated on Ficoll-Paque (1077 g/ml; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by centrifugation for 20 min at 1000g. The upper fraction was collected and washed in PBS. Isolated adMSCs were cultivated in DMEM medium (GIBCO cell culture media, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 5% HEPES, and 1% penicillin-streptomycin (all from Merck, Darmstadt, Germany), 1% GlutaMAX, 1% insulin-transferrin-selenium (both from GIBCO cell culture media, Thermo Fisher Scientific Inc., Carlsbad, CA, USA), epidermal growth factor (concentration 50 ng/ml; R&D Systems, Minneapolis, Minnesota, USA) at 37 °C in humidified incubator with 5% CO₂. Medium was changed twice a week and cells were passaged at 95% confluency until passage 6.

3.3. AdMSC classification

A cell suspension for testing was obtained by trypsinizing adMSCs (0.05% trypsin-EDTA solution; Merck, Darmstadt, Germany) and filtration through a 50-µm strainer in flow-cytometry washing solution (phosphate-buffered saline, 0.2% fish skin gelatin, 0.01% sodium azide;

Merck, Darmstadt, Germany). Aliquots from each single adMSC set were stained with the following antibodies: anti-mouse endoglin/CD105 (biotin labeled; R&D Systems, Minneapolis, Minnesota, USA), anti-mouse/rat CD29 (APC labeled; BioLegend, San Diego, California, USA), anti-rat/mouse CD90.1 Thy-1.1, (FITC labeled; eBioscience, Thermo Fisher Scientific Inc., Carlsbad, CA, USA) and anti-rat CD45 (PE-Cy5 labeled; BD Bioscience, San Jose, California, USA) and were incubated for 20 min. Subsequently, biotin was detected with Qdot 605 Streptavidin Conjugate (Invitrogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA). To differentiate the live and dead cells, samples were stained with Hoechst 33258 (Merck, Darmstadt, Germany) and analysed by flow cytometry (BD LSR II, BD Biosciences, San Jose, California, USA). The data were analysed using FlowJo software (BD Biosciences, San Jose, California, USA). AdMSCs were identified as a heterogeneous population of CD45-negative cells positive for CD29 and CD90 and partially positive for CD105 [23].

A Rat Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, Minnesota, USA) was used to determine the adMSCs' ability to differentiate into bone, fat, and cartilage, using the manufacturer's protocol. After differentiation, specific antigens were detected by immunofluorescence (adipocytes: anti-FABP4; osteoblasts: anti-osteocalcin; chondrocytes: anti-aggrecan).

3.4. Detection of vascular endothelial growth factor (VEGF) production

An adMSC suspension of 50,000 cells per 1 ml was placed into a 24-well culture plate and cultivated for 24 h at 37 °C in 5% CO₂. Next, a 300-µl sample of supernatant was taken from each well, frozen, and stored until analysed with the commercial Rat VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, Minnesota, USA).

3.5. Isolation of rat splenocytes

Spleens from the Lewis rats were disintegrated through a 500-µm strainer in Iscove's medium (Merck, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Merck, Darmstadt, Germany), 1% penicillin-streptomycin-glutamine (Merck, Darmstadt, Germany), and 0.4% heparin (Zentiva, Prague, Czech Republic). Splenocytes were separated on Ficoll-Paque (1077 g/ml gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by centrifugation for 30 min at 510g. The upper fraction was collected and washed in supplemented Iscove's medium. Isolated splenocytes were cryopreserved in Iscove's medium supplemented with 20% fetal bovine serum and 10% dimethyl sulphoxide (Merck, Darmstadt, Germany) and stored in liquid nitrogen until used.

3.6. Cultivation of endothelial cells

Lewis rat primary aortic endothelial cells (rat ECs) (cat. no. RA-6052LS, Cell Biologics, Chicago, IL, USA) and GFP-Expressing Human Brain Microvascular Endothelial Cells (human ECs) (Angio-Proteomie, Boston, MA, USA, cat. no. cAP-0002GFP) were cultured on 1% gelatin-coated flasks in Endothelial Cell Medium with Supplement kit (Cell Biologics Inc., Chicago, IL, USA) at 37 °C in 5% CO₂. The cytoplasmic uptake of acetylated low-density lipoprotein, Alexa Fluor 488 conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) was measured, and immunofluorescence labeling with anti-von Willebrand factor antibody (Dako, Agilent, Santa Clara, CA, USA) was performed to confirm the endothelial-specific properties of the cells.

3.7. Splenocyte proliferation assay

The proliferative response of freshly thawed Lewis rat splenocytes to human ECs in the presence or absence of Lewis rat adMSCs was assessed by splenocyte proliferation assay. For quantification of the proliferation rate, the Click-iT EdU Proliferation Assay for Microplates (Invitrogen,

Thermo Fisher Scientific, Carlsbad, CA, USA) was used according to the manufacturer's instructions.

Before the splenocyte proliferation assay, adMSCs and human ECs were incubated with mitomycin C (0.5 mg/ml in culture medium, 30 min at 37 °C; Merck, Darmstadt, Germany) and washed.

Cells were mixed in Iscove's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptavidin-glutamine and IL-2 (10 U/ml; Peprotech EC, Thermo Fisher Scientific, Carlsbad, CA, USA). We tested 5 different cell combinations:

- 1) 150,000 splenocytes + 5 µg/ml PHA (phytohemagglutinin A, Merck, Darmstadt, Germany).
- 2) 150,000 splenocytes + PHA + 10,000 adMSCs.
- 3) 150,000 splenocytes + 15,000 human ECs.
- 4) 150,000 splenocytes + 15,000 human ECs + 10,000 adMSCs.
- 5) 150,000 splenocytes alone.

Ninety-six-well plates were seeded with the cell combinations in 3 replicates and cultivated at 37 °C in 5% CO₂. After 4 days, 50 µl samples per well were aspirated and replaced with 50 µl of medium supplemented with 10 µl/ml EdU (the nucleoside analog 5-ethynyl-2'-deoxyuridine, included in the assay kit). After 3 days the incorporated EdU was detected according to the manufacturer's instructions. Briefly, the medium was removed and the cells were fixed for 5 min with 50 µl Click-iT EdU fixative and washed. The Click-iT reaction cocktail was added for 30 min to covalently join the horseradish peroxidase to EdU. After removing the supernatant, a 1.5% solution of bovine serum albumin (Merck, Darmstadt, Germany) in PBS was added for 5 min as a blocking solution. The wells were then washed 3 times. The Amplex UltraRed reaction mixture was added and incubated for 15 min. The conversion of Amplex UltraRed by horseradish peroxidase to a highly fluorescent product was stopped by adding Amplex UltraRed stop solution. The signal from the highly fluorescent product was recorded by using a fluorescence microplate reader (Synergy 2, BioTek, Agilent, Santa Clara, USA), using excitation at 568 nm and emission at 585 nm.

The response of the rat splenocytes was expressed as a stimulation index (SI): the ratio of the proliferation signal from the stimulated cells (combinations no. 1, 2, 3, or 4) to that from the splenocytes alone (no. 5, see schedule in text above).

3.8. Transplantation of adMSCs and GFP-expressing human ECs beneath the renal capsule

We tested the ability of adMSCs to decrease the xenoreactivity to human ECs in vivo after transplantation. Transplantation was performed under general anesthesia. Either 1×10^6 human ECs alone or 1×10^6 human ECs mixed with 2×10^6 adMSCs were transplanted under the kidney capsule of Lewis rats ($n = 6$). Briefly, after dorsolateral incision of the skin and muscle, the left kidney was exposed, a small incision in the renal capsule was made, and the cells were slowly injected through a 24G catheter (Becton Dickinson and Company, Franklin Lakes, USA).

3.9. Pancreas decellularization

The method of decellularization of a pancreatic tail has been described in detail recently [16]. In brief, the donor pancreas was cannulated via the splenic vein and perfused by a continuous recirculation system with 1% Triton X-100, followed with 0.5% sodium dodecyl sulfate and then again with 1% Triton X-100 (both from Glenthams Life Sciences Ltd., Corsham, UK). Finally, the organ was perfused with DNase solution (0.4 U/l; Merck, Darmstadt, Germany) and washed with PBS supplemented with 1% antibiotic antimycotic solution (Merck, Darmstadt, Germany). The pancreatic extracellular skeleton was stored in supplemented PBS at 4 °C until transplantation.

3.10. Isolation of pancreatic islets

Pancreatic islets were isolated by intraductal infusion of collagenase

(1 mg/ml; Merck, Darmstadt, Germany) [24]. Islets were separated from exocrine tissue on a discontinuous Ficoll gradient (Merck, Darmstadt, Germany). Isolated islets were cultivated in CMRL-1066 medium supplemented with 10% fetal bovine serum, 1% HEPES, and 1% penicillin-streptomycin (all reagents from Merck, Darmstadt, Germany) and 1% GlutaMAX (GIBCO cell culture media, Thermo Fisher Scientific, Carlsbad, CA, USA) at 37 °C in humidified incubator with 5% CO₂.

3.11. Transplantation of repopulated pancreatic matrix

Decellularized pancreatic skeletons were repopulated via the splenic vein by use of an indwelling cannula. Four freshly isolated pancreatic islets per 1 g of recipient body weight were infused in group A: alone ($n = 3$), in group B: together with 10×10^6 adMSCs ($n = 4$), or in group C: together with both 10×10^6 adMSCs and 5×10^6 rEC ($n = 3$) suspended in 0.2–0.5 ml of endothelial cell medium. Reseeded pancreatic skeletons were transplanted under general anesthesia into the dorsolateral subcutaneous space in Lewis rats. The size of the subcutaneous incision was approx. 1.5 cm and corresponded to the size of the recellularized matrix of the pancreatic tail.

3.12. Histological examination

The recipients were sacrificed under general anesthesia 9 days after transplantation. The timing of harvesting was based on our previous study, which examined the rate of revascularization of the pancreatic islet transplantation site [22,25]. The kidneys with transplanted GFP-human ECs with or without adMSCs were excised and the part containing the transplanted cells was dissected. The transplanted pancreatic skeletons were also excised from the subcutaneous space. Tissue samples were fixed in freshly prepared 4% buffered formaldehyde overnight at 4 °C, dehydrated, and embedded in paraffin. The parts of kidneys with transplanted GFP-hEC were embedded in Tissue-Tek O.C.T Compound (Sakura Finetek, Torrance, USA), frozen in liquid nitrogen, and stored at –80 °C.

3.13. GFP detection

Sections from frozen samples were counterstained with DAPI and embedded in DABCO-Mowiol solution (both from Merck, Darmstadt, Germany). The sections were examined with the use of a fluorescence microscope (Olympus BX41, Evident Europe GmbH, Hamburg, Germany).

3.14. Immunohistochemical antigen detection

Paraffin-embedded tissue was cut into 4-µm sections, which were deparaffinized and rehydrated. The sections were pre-treated using heat-mediated antigen retrieval with citrate or Tris buffer according to the manufacturer's recommendation (Vector Laboratories, Newark, USA) for 20 min. Endogenous peroxidase was blocked by 3% H₂O₂ in methanol for 20 min. To prevent nonspecific binding, samples were pre-incubated with 10% normal goat serum (Abcam, Cambridge, UK) in PBS with 0.05% Tween (Merck, Darmstadt, Germany). The sections were then incubated with primary antibodies overnight at 4 °C. We used the following antibodies: rabbit anti-Insulin (ab181547, dilution 1:40,000, citrate buffer for antigen retrieval; Abcam, Cambridge, UK), rabbit anti-CD4 (ab237722, dilution 1:1000, tris buffer for antigen retrieval; Abcam, Cambridge, UK), mouse anti-CD8 (ab33786, dil. 1:1000, citrate buffer for antigen retrieval; Abcam, Cambridge, UK), rabbit anti-CD20 (ab64088, dil 1:100, citrate buffer for antigen retrieval; Abcam, Cambridge, UK), mouse anti-rat monocytes/macrophages (MAB1435, dil. 1:100, tris buffer for antigen retrieval; Millipore, Temecula, CA, USA) and rabbit anti-CD31 (ab185981, 1:1000, Tris buffer; Abcam, Cambridge, UK). As controls we used Rabbit IgG or Mouse IgG₁ Isotype Control (14–4616-82 and 02–6100 resp., Thermo Fisher Scientific Inc.,

Carlsbad, CA, USA). A goat anti-rabbit IgG or horse anti-mouse IgG peroxidase-labeled secondary antibody (PI-1000 and PI-2000-1 resp., dilution 1:300, 1 h incubation at room temperature; Vector Laboratories, Newark, USA) was used to detect the primary antibody. A DAB Substrate Kit (ab64238, dilution 1:100, 4 min at RT; Abcam, Cambridge, UK) was used as the chromogen. Sections were then counterstained with Mayer's hematoxylin (DiaPath S.P.A. Martinengo, Italy), dehydrated, and mounted with Pertex (Histolab Products AB, Askim, Sweden). The sections were examined with an Olympus BX41 microscope and images were obtained with an Olympus DP71 camera (both from Evident Europe GmbH, Hamburg, Germany).

3.15. CD31-positive area measurement

Immunohistological images were imported into Corel Paint Shop Pro Photo X2 (Corel Corporation, Ottawa, Canada) to mark off the insulin-positive (pancreatic islets) and CD31-positive (endothelium) areas of subcutaneously transplanted repopulated skeletons. The pixel numbers in the selected areas were determined using ImageJ software (National Institutes of Health, Bethesda, USA), and the percentage of CD31-positive area within the islets was calculated.

4. Results

4.1. Characterization of adMSCs

The percentage of cells expressing specific MSC markers in the final preparations of adMSCs isolated from retroperitoneal and epididymal fat were as follows: CD29 88.2%–99.0% (mean \pm SD 94.6 ± 4.6), CD90 78.5%–98.0% (92.2 ± 8.9), CD105 41.1%–76.0% (64.2 ± 13.3). All 3 MSC markers were positive in the range of 33.8%–73.1% (56.2 ± 15.1) among the all live cells in the samples. The mean values were calculated from measurements of 6 samples.

The multipotency of isolated adMSCs was proved by their differentiation into chondrocytes, osteoblasts, and adipocytes using a Rat Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, USA) (Fig. 1).

The adMSCs in culture produced VEGF. The concentration of VEGF in the medium after 24-h culture of 50,000 adMSCs was 167–3220 pg/ml. The mean \pm SD of measurements of 8 samples was 1226 ± 1092 pg/ml.

4.2. Splenocyte proliferation assay

The modulatory effect of adMSCs was confirmed by splenocytes proliferation assay. The splenocytes' proliferative reaction to PHA was significantly decreased ($P = 0.03$, Student *t*-test, $n = 5$) in the presence of adMSCs. The splenocyte stimulation index values without and with adMSCs were 1.7 ± 0.51 (mean \pm SD) and 0.9 ± 0.47 (mean \pm SD), respectively (Fig. 2A).

The splenocytes' proliferative reaction to human ECs was also

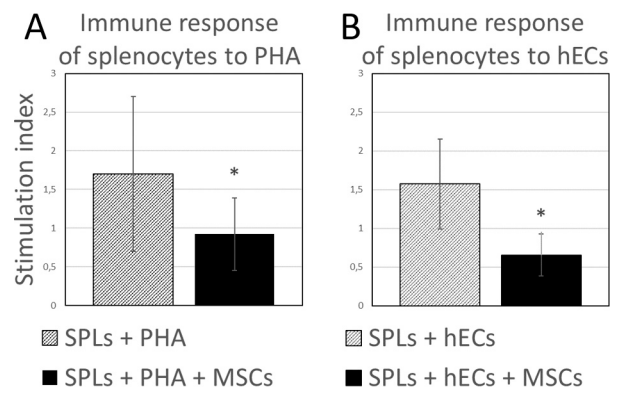


Fig. 2. Splenocyte proliferation assay. The proliferation is expressed as the stimulation index of splenocytes stimulated with (A) PHA and (B) human ECs. The addition of adMSCs significantly decreased splenocyte proliferation in response to both PHA and human ECs.

significantly decreased ($P = 0.008$, Student *t*-test, $n = 3$) in presence of rat adMSCs. The stimulation index values of splenocytes without and with adMSCs were 1.6 ± 0.58 (mean \pm SD) and 0.7 ± 0.27 (mean \pm SD), respectively (Fig. 2B).

4.3. Transplantation of adMSCs and human ECs beneath the renal capsule

Examination of dissected parts of kidneys following implantation of GFP-expressing human ECs did not reveal any fluorescence, suggesting their complete absence 9 days after implantation (Fig. 3A). We presume their complete destruction by the host immune system as the presence of these cells can be easily detected by their green fluorescence (Fig. 3C). Co-transplantation of syngeneic adMSCs was not able to ameliorate their rejection, given that no green fluorescence was detected (Fig. 3B). For this reason, we did not use this cell line in subsequent revascularization experiments.

4.4. Transplantation of adMSCs and rat ECs and pancreatic islets into the pancreatic extracellular matrix

4.4.1. Group A (islets alone)

Fig. 4 demonstrates a typical example of islets implanted into the pancreatic matrix excised 9 days after transplantation into the subcutaneous space. Insulin-positive areas are present within the skeleton; however, the cells show marks of disintegration. The low number of CD31-positive endothelial cells suggests that only very limited revascularization has occurred. This agrees with the rather low extent of CD31 positivity as detected by digital image analysis (see below). The experiments in all 3 animals show that the revascularization of the subcutaneously placed pancreatic matrices repopulated with pancreatic islets alone was insufficient.

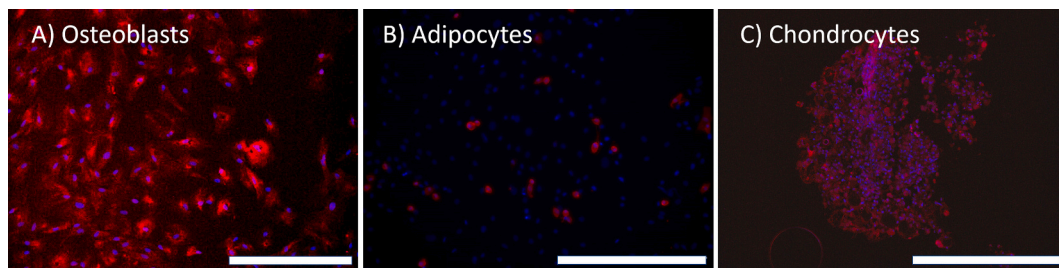


Fig. 1. AdMSCs differentiated in vitro. (A) Osteoblasts identified by anti-osteocalcin antibody (red), (B) adipocytes identified by anti-FABP4 antibody (red), and (C) chondrocytes identified by anti-aggrekan antibody (red). Cell nuclei were counterstained with DAPI (blue). Bars represent 400 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

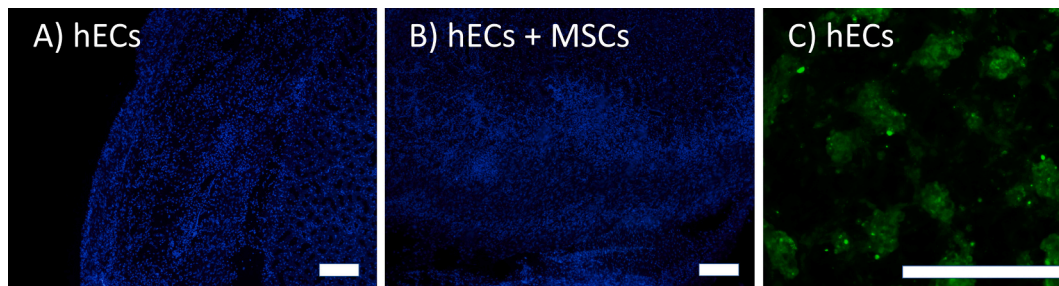


Fig. 3. Dissected parts of kidneys 9 days after the transplantation of human GFP-expressing human ECs. No GFP signal was detected in any examined sections. (A) human ECs transplanted alone, (B) human ECs transplanted together with rat adMSCs, (C) live GFP-expressing human ECs in vitro. GFP, green; cell nuclei, blue; bar represents 400 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

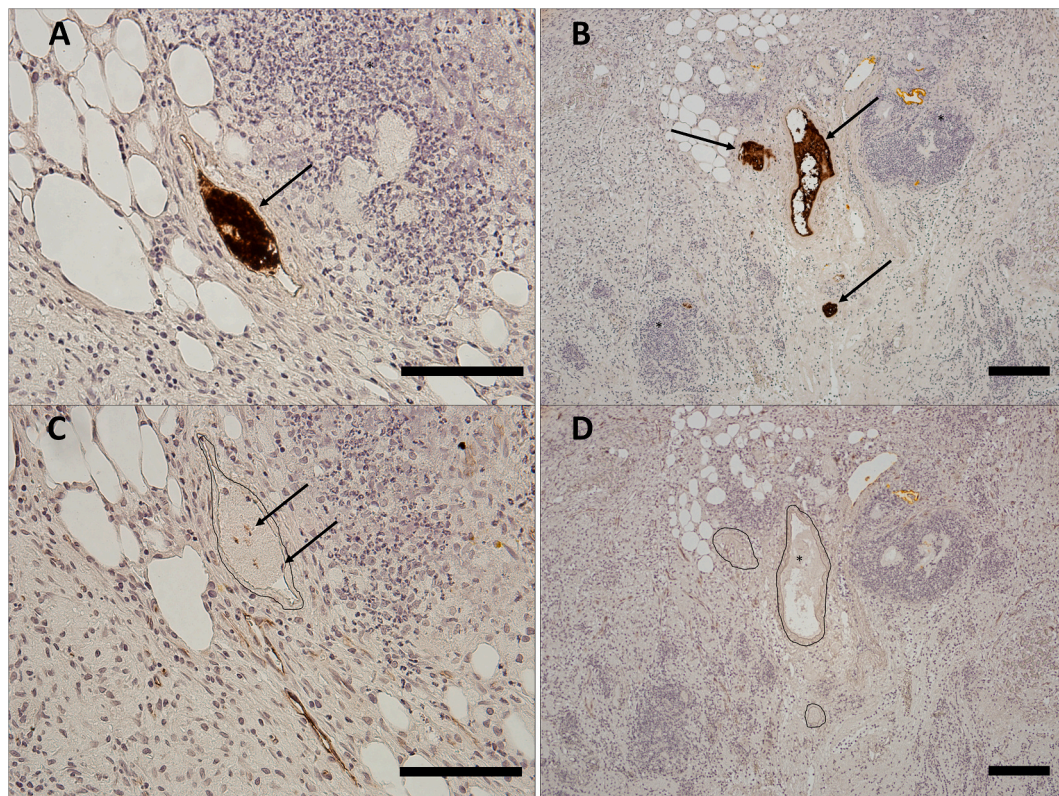


Fig. 4. Group A - pancreatic islets alone implanted into the pancreatic decellularized skeleton at 9 days after transplantation into the subcutaneous space. (A) and (B) Implanted islets detected by antibody labeling of insulin (brown, arrows); (*) immune infiltration in surrounding area. (C) and (D) Endothelial cells detected by anti-CD31 antibody (brown, arrows). The tissue in the area corresponding to the occurrence of the insulin shows signs of cell degradation (*). The area marked in black was used for EC quantification; hematoxylin counterstain, bars represent 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.4.2. Group B (islets together with adMSCs)

The pancreatic decellularized skeletons repopulated with islets and adMSCs 9 days after transplantation are shown in Fig. 5. Pancreatic islets containing insulin-positive beta cells were integrated within the surrounding tissue with almost no immuno-cellular infiltration. CD31 staining proved the abundant presence of endothelial cells, suggesting active revascularization of the implanted islets. In comparison with the previous group, the addition of adMSCs promoted revascularization and led to a better integration of the islets within the matrix in all 4 animals. The area of CD31-positive cells was also larger than in other experimental groups (see below).

4.4.3. Group C (islets together with adMSCs and rat ECs)

An example of the pancreatic decellularized skeletons repopulated by islets with both adMSCs and rat ECs is shown in Fig. 6. In contrast to

the result of transplantation of the islets with only adMSCs, cellular infiltration and necrotic cellular remnants surrounding the insulin-positive islet areas are present 9 days after transplantation. Beta cells were entrapped within the space corresponding to the decellularized veins of the pancreatic skeleton. CD31-positive endothelial cells were rarely detected in areas of insulin-positive cells. Similar findings in all 3 experimental animals suggest a rather unfavorable effect of the cell combination including the rat ECs.

4.4.4. Analysis of the CD31-positive area

Analysis of the CD31-positive area showed a positive effect of the addition of adMSCs to the pancreatic islet graft (group B) as compared with a graft without any cellular supplement (group A). Interestingly, the combination of adMSCs and rat ECs (group C) showed a rather negative effect.

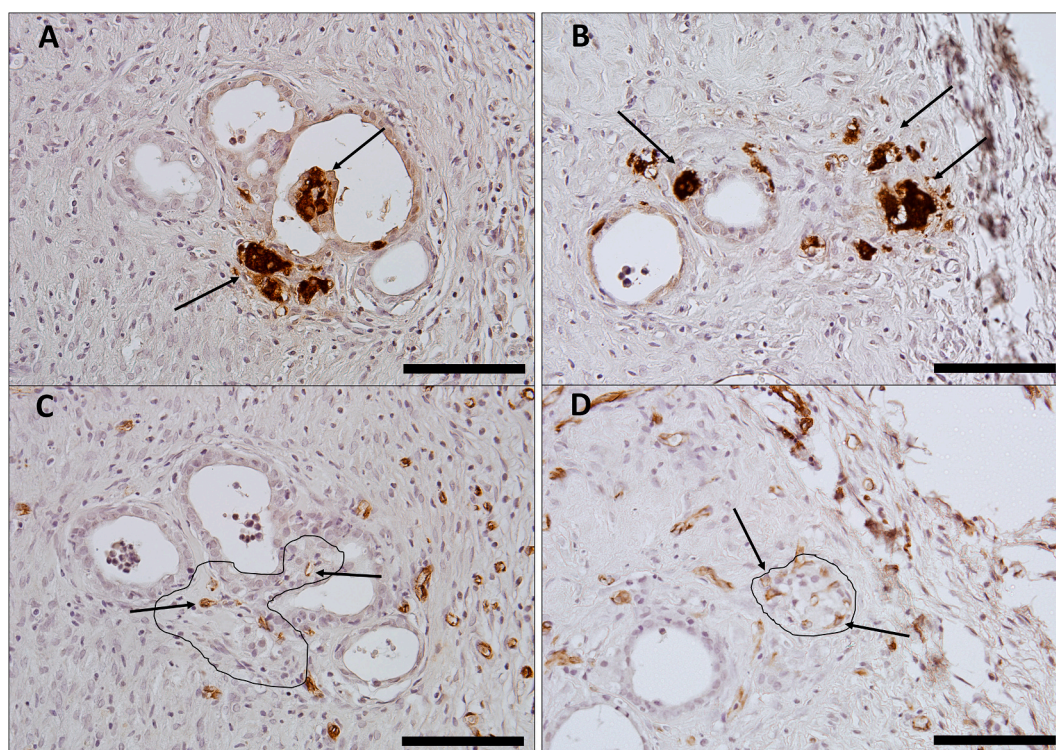


Fig. 5. Group B - pancreatic islets implanted into the pancreatic decellularized skeleton together with adMSCs at 9 days after transplantation into the subcutaneous space. (A) and (B) Implanted islets detected by antibody labeling of insulin (brown, arrows). (C) and (D) ECs detected by anti-CD31 antibody (brown, arrows) within the area corresponding to the occurrence of insulin and in surrounding tissue. The area marked in black was used for the quantification of ECs; hematoxylin counterstain, bars represent 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

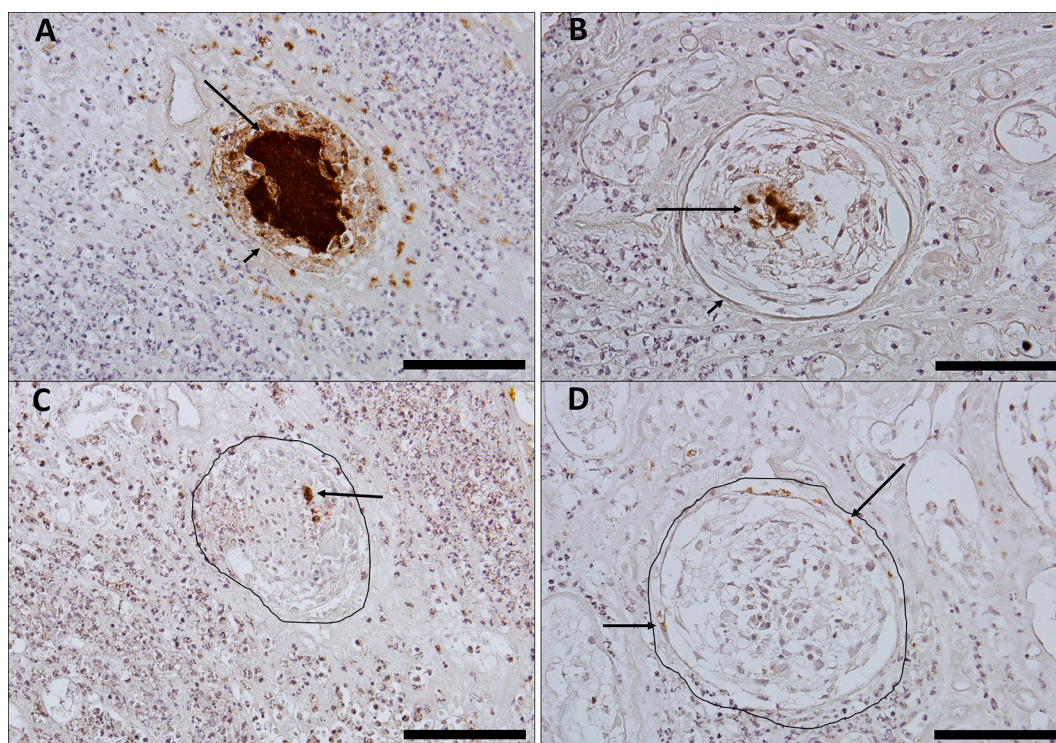


Fig. 6. Group C - pancreatic islets implanted into the pancreatic decellularized skeleton with adMSCs and rat ECs at 9 days after transplantation into the subcutaneous space. (A) and (B) Implanted islets detected by antibody labeling of insulin (brown, arrows), pancreatic islets entrapped inside the structures of decellularized vessels (arrow head), and immune infiltration in the surroundings. (C) and (D) Endothelial cells detected by anti-CD31 antibody (brown, arrows) with low abundance of ECs. The area marked in black was used for quantification of ECs; hematoxylin counterstain, bars represent 100 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The mean (\pm SD) percentage of CD31-positive area within the islets in the group A (islets alone), group B (islets plus adMSCs), and group C (islets plus adMSCs together with rat ECs) were $0.6\% \pm 0.8\%$, $4.5\% \pm 3.1\%$, and $1.0\% \pm 1.1\%$, respectively ($P < 0.05$; Student *t*-test); see Fig. 7.

4.4.5. Immune cells detection in transplanted skeletons

Histological staining of transplanted skeletons showed intensive cell infiltration in all groups. The immunodetection of specific immune cells didn't confirm lymphocytic infiltration. The B lymphocytes were not detected (Fig. 8A) as well as CD4+ T lymphocytes (Fig. 8B). Cytotoxic CD8+ T lymphocytes were detected only very rarely (Fig. 8C). Conversely, the massive macrophage infiltrations were detected in almost the whole transplanted skeletons both in groups A (Fig. 8D) and group C (not shown) but also in group B (Fig. 8E). Infiltration of polymorphonuclear cells was detected in the skeletons of groups A and C (Fig. 8F). In group B, polymorphonuclear cells were rare in vicinity of transplanted islets. The specificity of immunolabeling was confirmed by using of isotype control antibodies that gave no signals in transplanted skeletons (Fig. 9).

5. Discussion

The use of bioscaffolds constructed from decellularized organs is an attractive approach to supporting the survival, function, and availability of transplanted cells and tissues [26]. While a well-designed bioscaffold may be a suitable biological, structural, and mechanical support, it lacks an immediate vascular supply for the delivery of oxygen and nutrients. As pancreatic islet cells are highly sensitive to hypoxia [27,28], slow revascularization of the islet graft represents one of the major barriers to transplantation. This is also the reason why, among the few studies that have focused on pancreatic matrix revascularization, there are only rare reports with at least partial success [29]. One is the paper by Yu et al. [30], who used complicated direct arterial and venous anastomoses of the skeletons to renal vessels.

Neof ormation of vascular structures was also demonstrated by our group in islet-loaded decellularized matrices of pancreatic tail transplanted into a highly vascularized omental flap [16].

In the current study however, the same model of islet implantation into a matrix of decellularized pancreatic segment completely failed when it was transplanted into the dorsal subcutaneous space in rats. The islet structures within the skeletons were disintegrated and endothelial

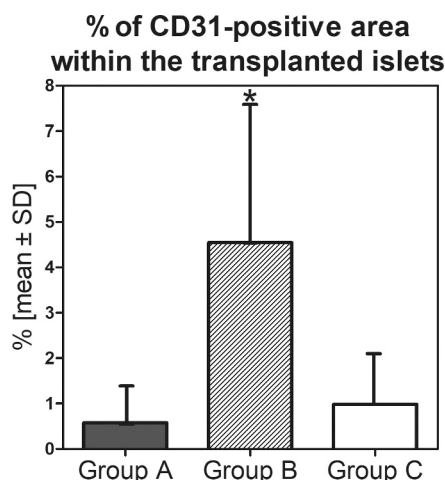


Fig. 7. Analysis of the CD31-positive area within the islets transplanted in skeletons. Expressed as % of CD31 positive pixels out of the pixels contained by the transplanted islet. Group A: islet alone, group B: islets plus adMSCs, Group C: islets plus adMSCs together with rat ECs; * denotes $P < 0.05$ versus other groups.

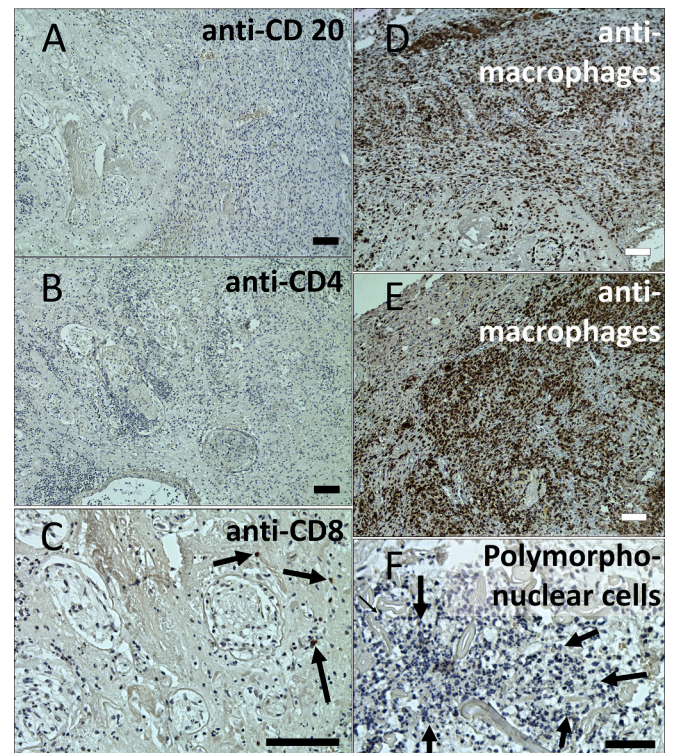


Fig. 8. Immunodetection of immune cells in transplanted skeletons at 9 days after transplantation into the subcutaneous space. Neither CD20+ B lymphocytes (8A) nor CD4+ T lymphocytes (8B) were detected in the skeletons. CD8+ T lymphocytes (arrows) were rarely detected (8C). Macrophages (brown) were abundant in transplanted skeletons in all groups; (8D) group A, islets only; (8E) group B, islets plus adMSCs. Polymorphonuclear cells (8F) were detected mainly in groups A and C (segmented nuclei in hematoxylin staining, arrows). Hematoxylin counterstain, bars represent 100 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells were rarely present. Therefore, we investigated several approaches to promote early vascularization using adipose tissue-derived mesenchymal stem cells (adMSCs) and endothelial cells (ECs).

First, we demonstrated that subcutaneous transplantation of pancreatic skeletons with islets alone led to islet cell necrosis, probably caused by hypoxia [27], accompanied by a non-specific inflammatory response manifested by cellular infiltration [31]. In our case, macrophages and polymorphonuclear cells predominated among the infiltrating cells. These cells play role mainly in innate immunity, acute inflammation and also in the elimination of debris and apoptotic cells [32].

On the other hand, co-transplantation of freshly isolated adMSCs significantly promoted early formation of endothelial structures and, at least in our short-term observation, enabled the survival of insulin-producing beta cells in the skeletons. Pancreatic islets were integrated within the pancreatic matrix of the skeletons, with positivity for insulin staining in the beta cells. Although macrophages were abundant in the skeletons, polymorphonuclear cells were almost not found around the islets transplanted with adMSCs. In addition, the presence of endothelial structures detected by immunohistochemical staining suggested a better early revascularization.

Even in this case, however, additional supplementation of the combination of islets and adMSCs with differentiated endothelial cells did not enhance revascularization. The revascularization of the skeletons was rather depressed and the skeletons were infiltrated with macrophages and polymorphonuclear cells. As we detected mainly the cells of innate immunity, we suggest that ECs were not destroyed by immune

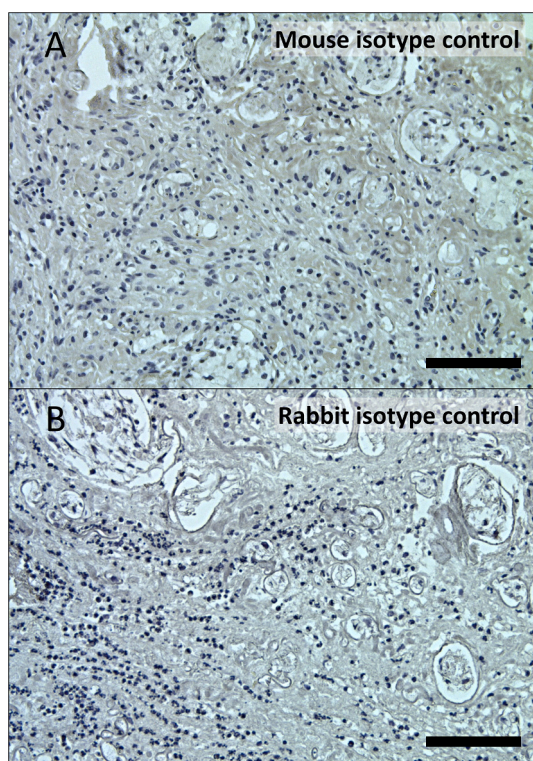


Fig. 9. Control staining with mouse and rabbit isotype controls. Negative staining confirmed specificity of antibody detection system; hematoxylin counterstain, bars represent 100 μm .

reaction but by hypoxia that can lead to their apoptosis and non-specific inflammatory response [33–35]. However, we couldn't proof this mechanism as the ECs were already eliminated in the transplanted skeletons of group C.

We are aware of the fact that studies have been published showing the benefit of adding endothelial cells to transplanted islets [36,37]. However, for example, Coppens et al. [38] showed that source of ECs was a very substantial. Human umbilical vein endothelial cells did not improve islet function in this study in contrast to human blood outgrowth endothelial cells. Also, in a paper by Aghazadeh et al. [39], the authors used rat endothelial cells which did not lead to significant improvement of vascularization. Better results were obtained with application of endothelial progenitors [40,41]. In our study, ECs had rather a harmful effect, that might have been caused by a low oxygen tension in the skeletons following a rather fast single dose repopulation. Therefore, cell administration into the skeletons using a slow infusion over several hours could be of advantage [42,43]. In our study, we used primary fully differentiated aortic endothelial cells. We admit that better results could be possibly achieved using a different ECs source.

We have also demonstrated the immunomodulatory potential of adMSCs in vitro, using a system Click-iT EdU. Proliferation of rat splenocytes stimulated by the non-specific T-lymphocyte mitogen phytohemagglutinin and by highly immunogenic human endothelial cells dropped significantly after the addition of rat adMSCs to the culture medium. As a proof of the immunomodulatory potential of MSCs, the mixed lymphocyte reaction or lymphocyte proliferative response to mitogens has been used in the past [44–47]. In our study, the traditional detection of incorporated ^3H -thymidine or BrdU [45,48] was replaced with the nucleoside analog EdU and Click-iT technology, which was originally developed as a tool to detect in vivo DNA synthesis [49], for cell cycle analysis [50,51], and to stain proliferating cells [52]. Quite recently, EdU was utilized to assess an equine xenogeneic mixed lymphocyte reaction [53]. In our study, we used this method for in vitro quantification of suppressive mesenchymal stem cell properties using

EdU for the first time.

A series of studies have demonstrated that co-culture or co-transplantation of mesenchymal cells with pancreatic islets improves islet function in vitro and after grafting, through various immunological and non-immunological mechanisms [54–57].

MSCs have also been used to repopulate various acellular scaffolds produced by decellularizing native organs, including lungs, trachea, urinary bladder, heart, heart valves, and others [58].

In general, MSCs have been shown to have the potential to differentiate into different cell types, to enhance angiogenesis, and to modulate the host immune reaction (to preserve fast degradation of the scaffolds), and in general they have shown promise in improving organ functionality and the biocompatibility of the scaffolds [58].

To our knowledge, there is only a single prior report on the use of MSCs to repopulate the decellularized pancreatic matrix [43]. Chandrika et al. used MSCs from human placenta to seed the decellularized mouse pancreas via the portal vein. Surprisingly, after placement of the repopulated grafts into the abdominal cavity close to the native pancreas in streptozotocin-diabetic mice, the authors reported functional recovery of both the endocrine and exocrine systems within the skeletons, leading to improvement of glucose tolerance after 20 days. Okcu et al. [59] studied the effect of co-culture of adMSCs together with alginate-encapsulated rat islets placed on a decellularized pancreatic matrix, but performed the experiments only in vitro. As in our study the recipients were non-diabetic, we did not follow glucose levels in the post-transplant period.

Our novel preliminary result is that the use of adMSCs supports faster islet engraftment in decellularized pancreatic matrices. Should the repopulated skeletons be transplanted into the subcutaneous space, into an omental flap or elsewhere, they could provide an alternative to direct intrahepatic islet implantation, avoiding the detrimental direct contact of islet cells with donor blood. MSCs are adult multipotent self-renewing cells able to differentiate into three mesodermal lineages—osteoblasts, adipocytes, and chondrocytes—in vitro [19,60,61], but also into endothelial cells [62]. In vivo, MSCs have been shown to secrete many types of cytokines and chemokines that affect ECs and induce angiogenesis [63]. This might be an advantage over the direct application of ECs, as shown by several previous studies [55,64,65] and in our current experiments. Besides, MSCs from adipose tissue are especially easy to isolate and standardize in high numbers [66] and, potentially, can be obtained from a specific tissue or organ recipient.

6. Conclusion

We have demonstrated that the addition of adipose tissue-derived mesenchymal stem cells improves early revascularization in decellularized pancreatic matrix seeded with pancreatic islets and transplanted into dorsal subcutaneous tissue in rats. Proof of the benefits of this procedure for islet function will require a longer follow-up and direct comparison with islet implantation into traditional sites. For testing of the modulatory effect of mesenchymal stem cells on the reactivity of splenocytes to foreign antigens in vitro, we have demonstrated for the first time the utility of the Click-iT EdU test.

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CRedit authorship contribution statement

Klára Zacharovová: Writing – original draft, Visualization,

Validation, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zuzana Berková:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Peter Girman:** Methodology, Data curation, Conceptualization. **František Saudek:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Data availability

Data will be made available on request.

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